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Award Number: DAMD17-99-1-9163

TITLE: Inhibition of Estrogen Receptor Action by the Orphan Receptors, SHP and DAX-1

PRINCIPAL INVESTIGATOR: James DiRenzo, Ph.D.

CONTRACTING ORGANIZATION: Dartmouth College
Hanover, New Hampshire 03755-1404

REPORT DATE: September 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 074-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | September 2002 | Annual Summary (1 Sep 01 - 31 Aug 02) |

**4. TITLE AND SUBTITLE**
Inhibition of Estrogen Receptor Action by the Orphan Receptors, SHP and DAX-1

**5. FUNDING NUMBERS**
DAMD17-99-1-9163

**6. AUTHOR(S)**
James DiRenzo, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Dartmouth College
Hanover, New Hampshire 03755-1404

E-Mail: **james.direnzo@dartmouth.edu**

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited.

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** ***(Maximum 200 Words)***

In support of DoD grant # DAMD17-99-1-9163, we present our progress towards understanding the function of mechanisms of action of two orphan nuclear receptors, SHP and DAX-1 as inhibitors of ERα and ERβ action. Consistent with prior studies of Shp, we show that Dax-1 binds directly to ERα and ERβ in an agonist dependent manner. In addition we show that this interaction is sufficient to abrogate transcriptional activation of reporter plasmids by ERa and ERb in a manner that is dose dependent. Mapping of this interaction showed that a 90 amino acid region of DAX-1 was sufficient to interact with agonist bound ERa and ERb. This region contains two LXXML motifs similar to the LXXLL motifs found in ligand-dependent co-activators of the SRC-1 family. Consistent with this, a peptide containing LHRLL from the second NR Box of GRIP one was able to compete away the interaction between ER and this 90 amino acid fragment. This suggests that the mechanism by which DAX-1 interacts with ERα and ERβ is similar to the interaction of agonist bound ER and the SRC-1 family of nuclear receptor co-activators. Additionally, over-expression of SRC-1 was sufficient to override the repression of ER activity by DAX-1. Taken together, these data support a model of ligand-dependent transcriptional repression by DAX-1 that is mediated by competition with and displacement of transcriptional co-activators of SRC-1 family.

| 14. SUBJECT TERMS | 15. NUMBER OF PAGES |
|---|---|
| Breast Cancer | 18 |
| | 16. PRICE CODE |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

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**Note to readers of this annual summary**

This award was initially made to Dr. Wongi Seol while she was a member of Dr. Myles Brown's laboratory at the Dana Farber Cancer Institute, and the manuscript which appears in the Appendix is the work of Dr. Seol. The transfer of this award to the current principle investigator, Dr.James DiRenzo, was made at the time that Dr. Seol discontinued her employment at the Dana Farber. Dr. DiRenzo has been the PI for this award since January of 2000, and has continued this work.

## Introduction

Lifetime exposure to estrogen is one of the most reliable predictors of breast cancer risk and is believed to underlie other risk factors such as age at menarche, age at first full-term pregnancy and possibly body-fat content. This fact coupled to the clear demonstration that estrogen dependent breast cancers can be chemo-prevented with Selective Estrogen Receptor Modulators (SERMS) such as tamoxifen provides abundant evidence for the critical role of estrogen signaling during initiation and progression of breast cancer. Therefore a greater understanding of the molecular mechanisms by which ER mediates the actions of estrogen continues to be a high priority within the field of breast cancer research. The elucidation of both the positive and negative regulation of ER function has and will continue to identify novel targets of therapeutic intervention.

In support of DoD grant number DAMD17-99-1-9163 we present our progress towards understanding the role of two orphan members of the nuclear receptor superfamily, Shp and DAX-1. Our findings suggest that these factors are capable of inhibiting the actions of ER through a physical interaction that is agonist dependent. Furthermore we show that this interaction is mediated by structural elements within SHP and DAX-1 that are highly similar to those found within several nuclear receptor co-activators including members of the Steroid Receptor Coactivator-1 (SRC-1) family. This finding has suggested a model in which Shp and DAX-1 are functioning as ligand-dependent co-repressors that physically compete with ligand-dependent co-activators for interaction with agonist bound ER. In support of this model we also show that the transcriptional repression that is mediated by Shp and DAX-1 is overcome by increased expression of SRC-1. This report will outline these findings and present relevant experimental data with specific reference to the original Statement of Work associated with this grant. Additionally current and future work that is directly related the Statement of Work will be described. Following this detailed description of our progress we will present, in a concise format Reportable Outcomes and a Modified Statement of Work.

## Original Statement of Work

**Inhibition of Estrogen Action by the Orphan Receptors SHP and DAX-1**

Task 1. To analyze interaction patterns with SHP-1 and DAX-1 with ERα and ERβ.

- A. Analysis of interaction patterns both *in vitro* and *in vivo*.
- **B.** Mapping of the interaction domains of both DAX-1 and ER
- **C.** Coimmunoprecipitation using extracts of either untransfected or transfected cell lines.

Task 2. To investigate the expression patterns of both SHP and DAX at both mRNA and protein levels in conjunction with ER levels.

- A. Isolation of mRNAs from human mammary epithelial cells and breast cancer cell lines.
- **B.** Analysis of RNA samples by northern blotting and/or quantitative RT-PCR
- **C.** Development of Anti-Shp1 monoclonal antibody.
- **D.** Analysis of protein levels of SHP and DAX-1.

Task 3. To study whether SHP or DAX-1 inhibits estrogen signaling.

- A. Effects of SHP or DAX-1 overexpression on ERα or ERβ in transient co-transfection.
- **B.** Effect of SHP or DAX-1 expression on the growth of ER positive breast cancer cell lines.

Task 4. Screen for SHP homologs expressed in breast tissue.

## Progress

**Task 1. To analyze interaction patterns with SHP-1 and DAX-1 with ERα and ERβ.**

**Analysis of interaction patterns both *in vitro* and *in vivo*.**

Studies of SHP and ER pertinent to Task 1 and Task 3 the interaction were have been published and a reprinted copy of this manuscript is attached in the appendix (Seol, Hanstein et al. 1998). In this manuscript we were able to show that there is a direct and ligand-dependent interaction between the hormone binding domain (HBD) of ER and SHP1. This interaction was demonstrated in vitro using the GST-pulldown assay. Briefly, a GST-ER-HBD fusion protein was produced in bacteria, extracted and immobilized on glutathione-linked agarose in the presence or absence of 17-β-estradiol or tamoxifen. These complexes were then used as affinity matrices to detect the interaction with $^{35}$S-SHP. Results indicated that SHP was capable of preferentially interacting with the HBD of ER in the presence of 17-β-estradiol but not tamoxifen. Similar experiments on deletion mutants of SHP demonstrated that a region spanning amino acids 92-148 was sufficient to mediate this interaction. In order to determine if this interaction occurred in vivo a mammalian two-hybrid system was devised in which the association of SHP-

VP16 with GAL4-ER HBD would activate a GAL4-luciferase reporter gene. These studies showed that SHP and ER were capable of interacting in vivo in response to estrogen. These studies also showed that SHP was capable of inhibiting ligand-dependent transcriptional activation of both ERα and ERβ which is described in Task 3.

**Mapping of the interaction domains of both DAX-1 and ER (Task 1B)**

The high level of conservation between SHP and DAX-1 coupled to the finding that DAX-1 was shown to be a repressor of the nuclear receptor SF-1 suggested that DAX-1 may function to inhibit estrogen action in a manner that was similar to that of SHP. To test this we first sought to determine if DAX-1 was capable of interacting with ER in an agonist-dependent manner. GST-ER HBD pulldown assays were performed in the presence or absence of 17-β-estradiol or tamoxifen and $^{35}$S-DAX-1. Results of this experiment suggested that DAX-1 interacts with ERα in an estrogen-dependent manner. In the presence of tamoxifen this interaction was reduced to levels below background suggesting that DAX-1 does not interact with ER in the presence of tamoxifen.

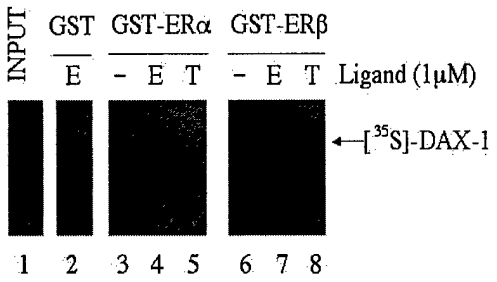


**Figure 1:** DAX-1 interacts with ERα in an agonist dependent manner. GST-ERHBD fusion proteins were incubated with radiolabelled DAX-1 in the Absence of estrogen or tamoxifen. Retained fractions were resolved on SDS-PAGE and autoradiographed. Results for ERα suggest an in vitro interaction that is Ligand dependent. Results for ERβ indicate preferential binding of DAX-1 in the Presence of estrogen as opposed to tamoxifen.

Having observed an interaction between ER and DAX-1, a series of deletion mutants of DAX-1 were generated and used to generate $^{35}$S-labelled products. Each of these products was subjected to the GST-ERHBD pulldown assay. The results of these experiments are summarized on Figure 2 (note the minimal interaction domain).

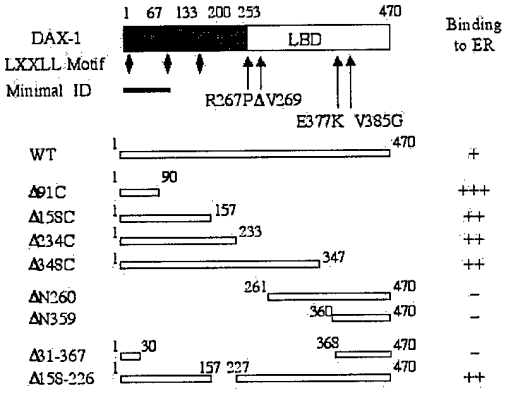


**Figure 2:** Summary of DAX-1 mutagenesis with regard to Estrogen-dependent interaction with ER. Results of these experiments indicate that the interaction between DAX-1 and ER is mediated by elements located within the first ninety amino acids.

Taken together, these studies suggest that DAX-1 is capable of interacting with ER in an agonist dependent manner and that this interaction is mediated by elements within the first ninety amino acids of DAX-1. The observation that SHP-1 appeared to compete with members of the SRC-1 family of nuclear receptor coactivators, suggested that DAX-1 may interact through similar elements. A visual inspection of the 90 amino acid interaction domain of DAX revealed two LXXML motifs similar to the LXXLL motifs identified as the minimal interaction domain for the ligand dependent co-activators of the SRC-1 family (Heery, Kalkhoven et al. 1997; Darimont, Wagner et al. 1998; Ding, Anderson et al. 1998). In order to test if the interaction between DAX-1 and ER was mediated through the LXXML sequences GST-pulldown assays were performed to test the ability of DAX-1 and ER to interact in a ligand-dependent manner in the presence of a peptide representing nuclear receptor box 2 (NR2) from the SRC-1 family member GRIP1. Results of these studies indicated that excess NR2 peptide effectively blocked the interaction between DAX-1 and ER while similar amounts of an NR2 alanine substituted mutant did not. These studies (Figure 3) suggest that DAX-1 interacts with ER through the LXXML sequences located in the first 90 amino acids of DAX-1.

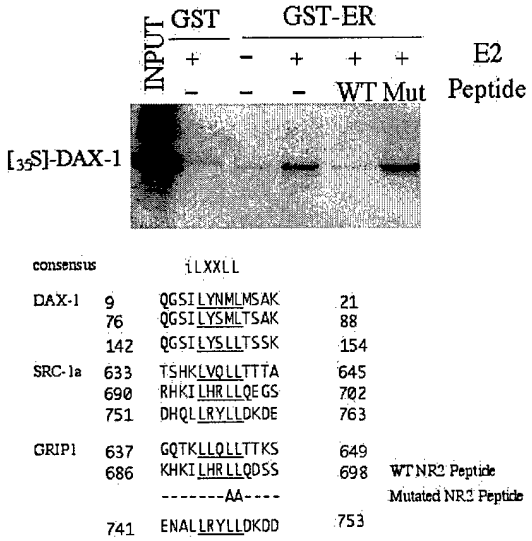


**Figure 3:** GST-ER HBD pulldown of radio-labeled DAX-1 is inhibited by NR2 of GRIP1. GST-pulldown assays were performed asdescribed above in the presence or absence of peptides representing the NR2 of GRIP1 or an alanine subsituted mutant. Retained fractions were resolved on SDS-PAGE and autoradiographed. Results show that while the NR2 WT peptide was an effective inhibitor of ER/DAX-1 interactions the alanine subsituted mutant was not.

The observation of a ligand-dependent interaction between ER and DAX-1 coupled to the earlier finding that SHP was capable of functioning as a repressor of ER activity predicted that DAX-1 would act

in a similar manner. To test this transient transfection assays were conducted to determine the effects of DAX-1 over-expression on estrogen signaling. Results (Figure 4) showed that hormone-dependent activation of an idealized estrogen reporter by either ERα or ERβ was potently repressed by DAX-1, and that this repression was does dependent. Similar results were also obtained in HEPG2 cells (not shown).

Several studies have shown that mutations in the gene encoding DAX-1 are responsible for Adrenal Hypoplasia Congenita (AHC) (Muscatelli, Strom et al. 1994). Several point mutations have been identified from affected patients (Peter, Viemann et al. 1998). Using these mutations we sought to determine if they would have any effect on the ability of DAX-1 to inhibit estrogen action. We first sought to determine if these mutations resulted in a change in the ability of DAX-1 to interact with ER in a hormone-dependent manner. To test this GST pull-down assays were performed as described using similar amounts of $^{35}$S-labeled proteins. Figure 5 shows that these mutants retained their ability to interact with ER and suggested that these mutations may disrupt the ability to repress transcriptional activation by ER. To test this wt DAX and AHC-derived mutant isoforms of DAX-1 were tested for their ability to repress ER-mediated transcriptional acitivity. Results (Figure 6) indicate that while the AHC-derived mutant isoforms of DAX-1 were capable of interacting with ER they were incapable of repressing ER-mediated transactivation. This result suggests that the mechanism by which DAX-1 represses the activity of ER and possibly other nuclear receptors is through the recruitment of additional factors.

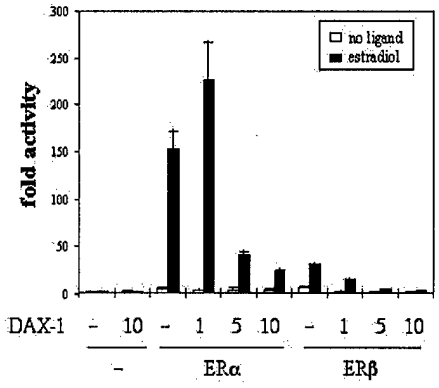


**Figure 4:** Trasnsient transfection of DAX-1 reveals a dose-dependent inhibition of estrogen receptor activity. U2OS cells were transiently transfected by the calcium phosphate method. Each transfection included the (ERE)-tk-luciferase reporter, the indicated ER expression plasmids (25 ng/well) and the indicated DAX-1 expression plasmid (25, 125 or 250 ng/well). Numbers indiacte the relative ratio of ER plasmid to DAX-1 plasmid. All experimental points were internally controlled by co-transfection of 25 ng/well of tk-beta-galactosidease. Data are expressed as the fold increase in response to estradiol after correction for galactosidase activity.

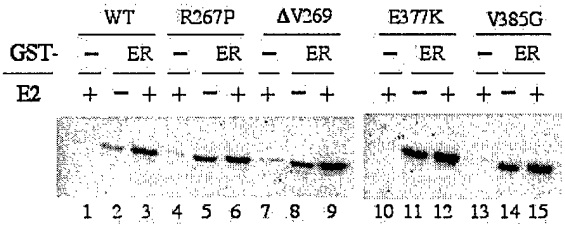


**Figure 5:** Clinically relevant mutants taken from AHC patients retain their ability to interact with ER in a hormone-dependent manner. GST-ER HBD pulldown assays were conducted with the indicated DAX-1 mutants. Results show that each of these mutants retains its ability to interact with agonist-bound ER.

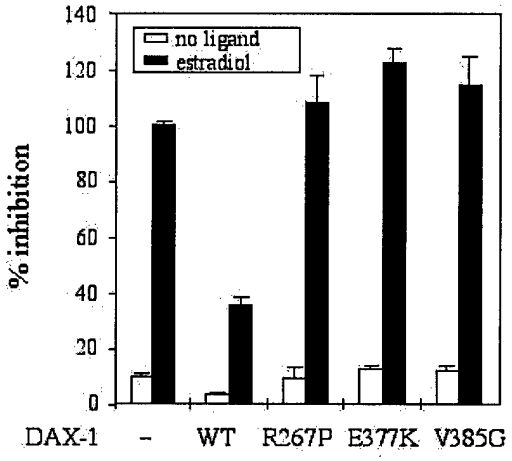


**Figure 6:** Over expression of AHC-derived mutants of DAX-1 fails to repress ER-mediated transcriptional activation. The indicated mutant isoforms of DAX-1 were subcloned into pcDNA and the resulting plasmids were transfected into U2OS cells as described. Shown are the results of a representative experiment in which wt DAX-1 but not R267P, E377K or V385G was sufficient to repress ER activity.

## Conclusions

The studies presented here and in the attached manuscript support the conclusion that SHP and DAX-1 are capable of acting as agonist-dependent co-repressors of the transcriptional activities of ERα and ERβ. In support of DoD grant # DAMD17-99-1-9163 we show that both SHP and DAX-1 are capble of interacting with ER in a manner that is dependent upon agonist binding, and that this interaction is inhibited by SERMs such as tamoxifen. Additionally we show that both SHP and DAX-1 utilize similar

structural elements that mediate the interaction with ER and that these elements overlap with the Nuclear Receptor Boxes (NR Boxes) identified in the SRC-1 family of ligand-dependent coactivators. This conclusion is supported by the finding that over-expression of SRC-1 was sufficient to abolish SHP-1 mediated inhibition of ER acitivity. In addition to these studies we have shown that mutant isoforms of DAX-1 derived from Adrenal Hypoplasia Congenita (AHC) retain their ability to interact with agonsit bound ER but lose their ability to repress ER-mediated transcriptional acitivation, suggesting that DAX-1 may be functioning to recruit other factors that may directly repress gene activation.

**Key Research Accomplishments**

A. Characterization of the interactions of SHP and DAX-1 with agonist-bound ER.
B. Identification and mapping of structural elements with SHP and DAX-1 that directly mediated the interaction with ER.
C. Demonstration that SHP and DAX-1 are capable of functioning as agonist-dependent co-repressors of ER activity.
D. Demonstration that the activity of DAX-1 as a co-repressor likely involved the recruitment of additional factors capable of repressing transcription.
E. Demonstration that the interaction between DAX-1 and ER can be specifically competed with peptides derived from the NR box of the ligand-dependent coactivator GRIP-1.
F. Functional demonstration that both SHP and DAX-1 are capable of blocking ER activity.

**Reportable Outcomes**

We have provided biochemical and genetic evidence for a regulatory mechanism that will inhibit estrogen receptor dependent transcriptional activity in the presence of estrogen. While the concept of an agonist-dependent co-repressor may initially seem paradoxical, we believe that such a mechanism may be critical in controlling cellular responses to estrogen. Some of our findings that are listed above in the "Key Research Accomplishments" section have been published in the attached manuscript while others are in preparation for publication.

We believe that these studies may be relevant to the initiation of hormone-dependent breast cancer and are currently working to establish non-transformed immortalized mammary epithelial cell model systems to determine if the activities of SHP and DAX-1 in a non-transformed system are consistent with what we have observed in transformed cells.

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# Inhibition of Estrogen Receptor Action by the Orphan Receptor SHP (Short Heterodimer Partner)

**Wongi Seol, Bettina Hanstein*, Myles Brown, and David D. Moore**

Department of Cell Biology (D.D.M.)
Baylor College of Medicine
Houston, Texas 77030

Department of Adult Oncology (W.S., B.H., M.B.)
Dana-Farber Cancer Institute
Boston, Massachusetts 02115

**SHP (short heterodimer partner) is an unusual orphan receptor that lacks a conventional DNA-binding domain. Previous results have shown that it interacts with several other nuclear hormone receptors, including the retinoid and thyroid hormone receptors, and inhibits their ligand-dependent transcriptional activation. Here we show that SHP also interacts with estrogen receptors and inhibits their function. In mammalian and yeast two-hybrid systems as well as glutathione-S-transferase pull-down assays, SHP interacts specifically with estrogen receptor-$\alpha$ (ER$\alpha$) in an agonist-dependent manner. The same assay systems using various deletion mutants of SHP map the interaction domain with ER$\alpha$ to the same SHP sequences required for interaction with the nonsteroid hormone receptors such as retinoid X receptor and thyroid hormone receptor. In transient cotransfection assays, SHP inhibits estradiol -dependent activation by ER$\alpha$ by about 5-fold. In contrast, SHP interacts with ER$\beta$ independent of ligand and reduces its ability to activate transcription by only 50%. These data suggest that SHP functions to regulate estrogen signaling through a direct interaction with ER$\alpha$. (Molecular Endocrinology 12: 1551–1557, 1998)**

## INTRODUCTION

The nuclear hormone receptor superfamily is a group of transcription factors that regulate target genes in response to small hydrophobic compounds such as steroids, thyroid hormone, and retinoids. The members of this superfamily play a variety of important roles in development and differentiation by regulating transcription of specific target genes (1–4). In general, these proteins bind DNA with high affinity as dimers. The majority of the type II or nonsteroid receptors, such as the thyroid hormone receptor (TR), retinoic acid receptor (RAR), or the orphan receptors, require heterodimerization with the retinoid X receptor (RXR) for high-affinity binding to their response elements (2). In contrast, the steroid receptors are generally thought to bind to their response elements exclusively as homodimers (*e.g.* Ref. 1), although the recently described ER$\beta$ isoform (5, 6) suggests the possibility of additional interactions.

Estrogen receptor (ER) regulates gene expression in female reproductive tissues in response to estrogen and is an important target in breast cancer therapy, as more than half of breast cancers express high levels of ER and depend on estrogen for growth. Although the ER$\alpha$ and ER$\beta$ isoforms recognize the same response elements, ER$\beta$ binds estrogen with a lower affinity than ER$\alpha$ and shows weaker transactivation activity (7). In addition, ER$\beta$ may activate a different spectrum of genes in response to certain ligands (8). Both the overlapping expression patterns of ER$\alpha$ and $\beta$ in various tissues and the possibility of formation of ER$\alpha$-ER$\beta$ heterodimers have complicated the understanding of gene regulation by estrogen. Furthermore, recent studies suggest that ER function may be modulated by cyclin D1 (9, 10) and that epidermal growth factor, transforming growth factor-$\alpha$, and dopamine (11) elicit estrogen-independent, ER-dependent gene transcription of estrogen-responsive genes. Thus, ERs may have functional interactions with a range of other signaling molecules.

SHP (short heterodimer partner) was initially isolated by yeast two-hybrid screening (12) based on its interaction with mCAR (13), the murine homolog of the orphan receptor MB67 (14). Isolation of full-length cDNA clones revealed that SHP, like the orphan DAX-1 (15), lacks a conventional DNA-binding domain (12). Both direct biochemical analysis and results from the yeast two-hybrid system demonstrated that SHP can

0888-8809/98/$3.00/0
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**Table 1.** Interaction of Full-Length SHP and Mutants with ER in Yeast

| | Lex– | Lex-SHP | | | | |
|---|---|---|---|---|---|---|
| | | WT | ΔE1 | ΔN-148 | ΔE1X | W160X |
| B42– | – | – | – | – | – | – |
| –ERα | –/– | +/++ | –/+ | +/++ | –/– | –/++ |
| –RXRα | –/– | ++/++ | +/++ | ++/++ | –/– | ++/++ |

++, Strongly blue colonies after 1 day of incubation and strong interaction; +, light blue colonies after 1 day incubation and weak interaction; –, white colonies and no interaction.
For RXR and ER, 100 μl of a $10^{-6}$ M stock of the appropriate ligand was added to the plate before plating, as previously described (30). Results are indicated in the absence and presence of ligand (–/+). The LBD of human ERα and human RXRα were used for construction of B42 fusions.

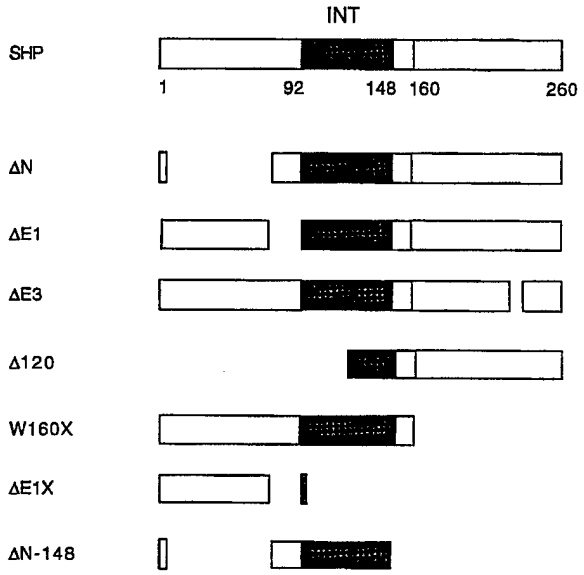


**Fig. 1.** Deletion Mutants of SHP
The ER-interacting domain is indicated by a *solid box*. The deletion constructs were previously described (16).

interact with the thyroid hormone receptor (TR), RAR, RXR, and other members of the receptor superfamily. In addition, SHP inhibits *in vitro* DNA binding by RAR/RXR heterodimers. In mammalian cell cotransfections, SHP also inhibits transactivation by RAR, mCAR, and other receptor superfamily members with which it interacts (12). These results suggest that the major physiological role of SHP is to repress signaling by other receptor superfamily members. Additional support for this hypothesis was provided by the recent demonstration that SHP itself can act as a direct transcriptional repressor (16). The receptor interaction and repression domains of SHP were mapped to the central region and carboxyl terminus, respectively, by both mammalian and yeast two-hybrid assays (16).

We have examined the potential role of SHP in estrogen signaling. Here we report that SHP interacts with ERα, both *in vitro* and in yeast and mammalian two-hybrid systems in a ligand-dependent fashion. As expected from its functional effects with other receptors, SHP inhibits transactivation by ERα induced by estrogen. Thus we propose that SHP is a novel inhibitor of ERα activity and may play a role in modulating cellular responses to estrogen.

## RESULTS

### SHP Interacts with ER

We have shown previously that SHP interacts with RXR and several of its heterodimer partners, such as RAR, TR, and mCAR, using two-hybrid, biochemical, and functional assays (12, 16). Unexpectedly, the same approaches demonstrate that SHP also interacts with human estrogen receptor α (ERα). In yeast, ERα interacts with full-length SHP (Table 1). The pattern of interaction of ER with a series of SHP deletion mutants (Fig. 1) previously used to identify SHP sequences required for interaction with other receptors (16) is quite similar to that described for RXR, although interaction of ER with the ΔE1 and W160X mutants is more ligand dependent (Table 1). These results identify SHP residues 72–148 as the minimal ER interaction domain. This same region is sufficient for interaction with RXR, TR, RAR, and mCAR (16).

The possibility of a direct interaction of SHP with ER was tested biochemically. As indicated in Fig. 2A, [$^{35}$S]methionine-labeled SHP showed 17β-estradiol-dependent interaction with a glutathione-*S*-transferase (GST)-human ERα ligand-binding domain (LBD) fusion protein (lanes 2–4). This SHP-ER interaction was as strong as or stronger than interaction of SHP with full-length RXR (lanes 3–6). An analogous, although somewhat weaker, ligand-dependent interaction was also observed between a GST full-length SHP fusion and [$^{35}$S]methionine-labeled full-length hERα (data not shown). The interaction of rat ERβ (rERβ) and SHP was also examined using GST-rERβ LBD and [$^{35}$S]methionine-labeled SHP. A specific, but ligand-independent and relatively weak interaction was observed (data not shown). In contrast to these results with ER isoforms, a GST-human androgen receptor LBD (GST-hAR) fusion protein did not interact with SHP in either the presence or absence of its ligand (Fig. 2A, lanes 7 and 8), and GST-SHP also showed no

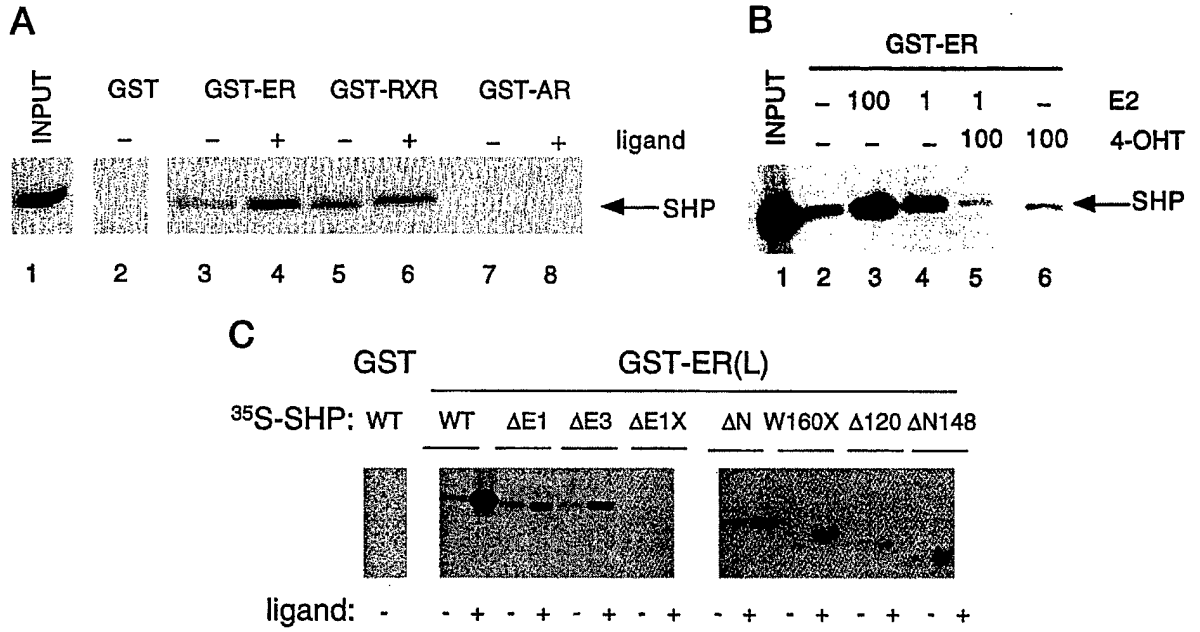


**Fig. 2.** *In vitro* Interaction of GST-Receptor Fusion Protein with SHP

Full-length murine SHP and various mutants (Fig. 1) were radiolabeled with [$^{35}$S]methionine by *in vitro* translation with pT7IacHisMycSHP. A, Specific interaction of SHP with GST-human ERα. GST-ERα LBD, -AR LBD, and -RXRα full-length proteins were expressed in *E. coli* BL21 strain and tested for interaction with SHP in the absence and presence of their cognate ligands (25). For ligands, 1 μM of 17β-estradiol, 9-*cis*-retinoic acid, and R1881 were used for ER, RXR, and AR, respectively, and indicated as +. SHP did not bind to GST protein. About 20% of SHP used in each lane was shown as INPUT (lane 1). B, Effect of 4-OHT on interaction of SHP with ER. GST-human ERα LBD fusion protein was incubated in the absence or presence of 10 nM (indicated as 1) or 1 μM (indicated as 100) 17β-estradiol ($E_2$). To test whether SHP can interact with ER in the presence of a partial agonist, 1 μM 4-OHT was incubated with or without 10 nM estradiol. About 20% of radiolabeled SHP used in each lane was shown as INPUT (lane 1). c, Mapping of ER-interacting domain of SHP. SHP products were tested for interaction with a GST fusion to LBD of human ERα in the absence and presence of 1 μM 17β-estradiol. Tests of SHP proteins with GST alone did not show any specific interaction.

interaction with radiolabeled full-length hAR (data not shown).

As 4-hydroxy-tamoxifen (4-OHT) acts as a partial ERα agonist in liver, where SHP is highly expressed, the effect of 4-OHT on the *in vitro* interaction between SHP and ERα was examined. As shown in Fig. 2B, SHP did not bind to ER in the presence of 1 μM 4-OHT (lane 6). Moreover, addition of 1 μM of 4-OHT abolished the hormone-dependent interaction observed in the presence of 10 nM estradiol (lane 5). These results indicate that SHP specifically recognizes the estrogen-bound form of ERα.

Various deletion mutants of SHP were used to confirm the mapping of the ER interaction domain of SHP (Fig. 2C). Consistent with the results observed in yeast, the pattern of *in vitro* interactions was almost identical to that reported for RXR (16). SHP-ER interaction was retained with several SHP mutants, including W160X, which lack the I-box or the 9th heptad, as well as internal deletions of either this region (ΔE3), or of the more highly conserved signature motif located near the N terminus of the LBD (ΔE1). The SHP N-terminal deletion Δ120 did not show specific interaction *in vitro* with GST-ER, relative to GST alone. The smallest protein retaining specific *in vitro* interaction was the ΔN-148 mutant, as observed in yeast (Fig. 2C and Table 1).

To further confirm these results, the interaction of SHP with ERα was tested using the mammalian two-hybrid system, with GAL4-hERα LBD and VP16-SHP fusion constructs. Cotransfection of GAL4-ER with VP16 alone in HepG2 cells showed approximately 5-fold activation by 17β-estradiol relative to cotransfection of both empty vectors, GAL4 and VP16. However, cotransfection of VP16-SHP fusion with GAL4-ER LBD increased transactivation to more than 35-fold, or 7-fold greater than that of the GAL4-ERα LBD with VP16 alone (Fig. 3). Very similar results were obtained in transfections using U2-OS cells (data not shown).

Overall, we conclude that SHP specifically interacts with ER in a ligand-dependent manner, as demonstrated by both yeast and mammalian two-hybrid systems, and by *in vitro* assays.

### SHP Inhibits Transactivation by ER

SHP inhibits transactivation by RAR, mCAR, and other nuclear receptors (12). Thus, the effect of SHP on transactivation by human ERα was tested in transient

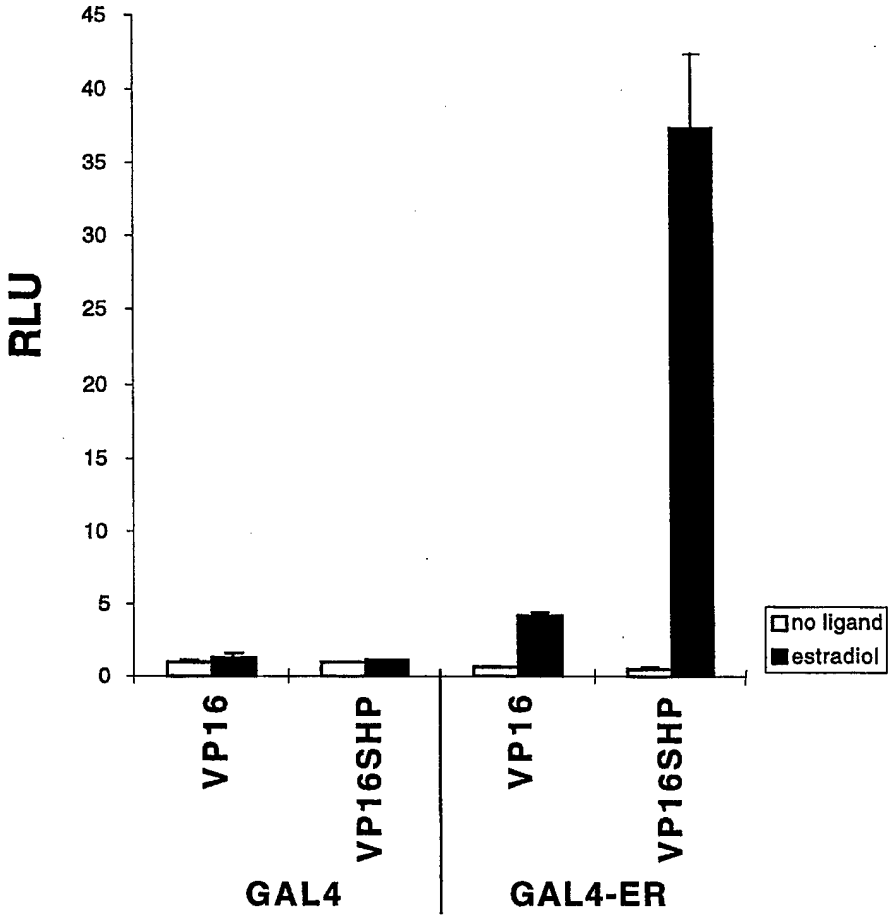


**Fig. 3.** Specific Interaction of ER with SHP in HepG2 Cells by a Mammalian Two-Hybrid Assay

GAL4-human ERα LBD (0.1 μg/well) and VP16-SHP (0.5 μg/well) were cotransfected by diethylaminoethyl-dextran method (28) with TKGH (0.5 μg/well) and luciferase reporter plasmid containing three copies of GAL4 DNA-binding element (0.5 μg/well) into HepG2 cells grown in 12-well plates. Fresh media containing either 1 μM 17β-estradiol or ethanol were added about 16 h after DMSO shock. Results are expressed relative to luciferase expression with empty vectors, GAL4 alone plus VP16, in the absence of ligand. The data were reproducible, and a similar pattern was obtained with transfection of U2-OS cells.

cotransfections using a luciferase reporter containing three copies of the vitellogenin estrogen response element (ERE). As shown in Fig. 4, A and B, this reporter was transactivated by approximately 30-fold by ERα in U2-OS cells. However, this transactivation was decreased in a dose-dependent manner by SHP coexpression, with only approximately 20% of the response remaining with a 10-fold molar excess of SHP expression vector (Fig. 4B). This level of inhibition is similar to that previously observed with mCAR and RXR, and stronger than that with RAR (12). As expected, a similar inhibitory effect of SHP on ERα was also observed in HepG2 cells (data not shown). Consistent with the relatively weak, ligand-independent interaction of SHP with ERβ observed *in vitro*, SHP coexpression decreased ERβ transactivation by approximately 50% in either the presence or absence of ligand (Fig. 4A).

4-OHT acted as a partial agonist with ERα but not ERβ in U2-OS cells. Somewhat unexpectedly, SHP also decreased this activation of ERα, although this effect was much weaker than that observed with estradiol- activated ERα. The basis for this inhibition is unknown, but it suggests that the partial agonist activity of 4-OHT in these cells may be due, in part, to a weak, but direct, activation of the ER LBD.

In striking contrast to the results with ERα, SHP showed no effect on transactivation of the mouse mammary tumor promoter (MMTV) promoter by the AR (Fig. 4C). This lack of effect is consistent with the lack of apparent *in vitro* interaction between SHP and AR (Fig. 2A) and clearly demonstrates that SHP is not a general inhibitor of all members of the nuclear hormone receptor superfamily.

## DISCUSSION

The results described here confirm and significantly extend previous indications that SHP can interact with

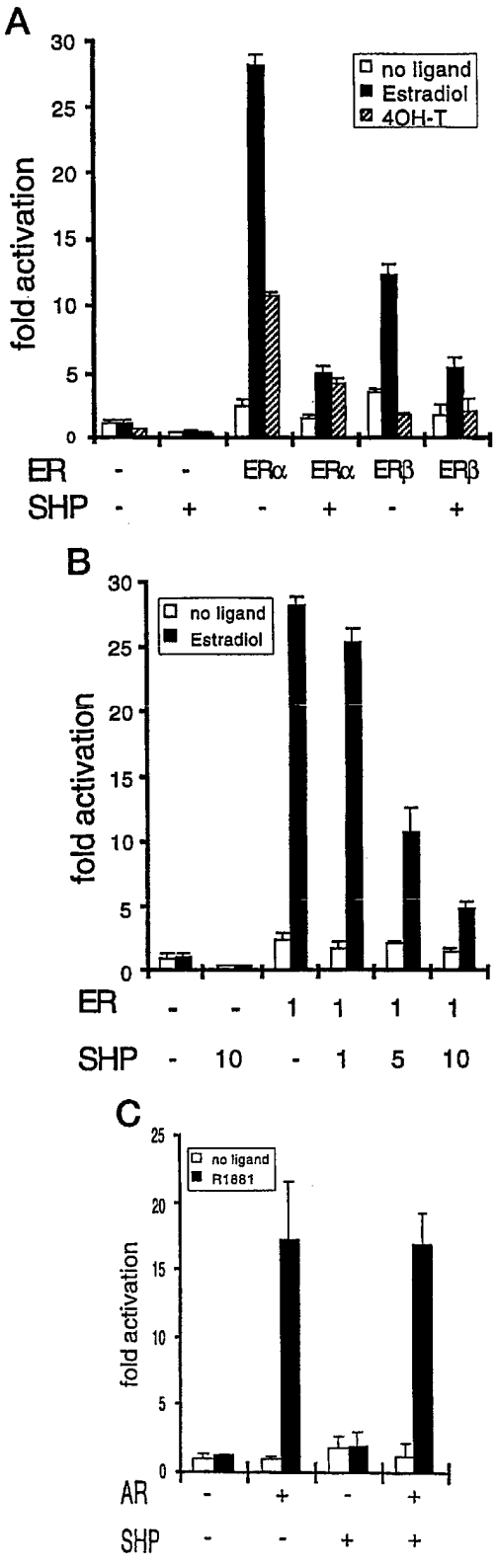


**Fig. 4.** Specific Inhibition of ERα by SHP

A, Inhibition of transactivation by ER with SHP. Plasmids containing human ERα and rat ERβ1 in pCDNA3 vector (25 ng/well) and SHP in CDM8 vector (250 ng/well) were cotransfected with TKβ-gal (125 ng/well) and ERETKluciferase (250 ng/well), a luciferase reporter plasmid containing three copies of vitellogenin ER response element, into U2-OS cells grown in 24-well plates. Results are expressed relative to luciferase activity with pCDNA3 alone without 17β-estradiol. The data were reproducible, and a similar pattern was obtained with transfection of HepG2 cells. B, Dose-dependent inhibition of SHP. Plasmids containing human ERα in pCDNA3 vector (25 ng/well) were cotransfected with 1-, 5-, or 10-fold concentration of SHP in CDM8 vector (25, 125, or 250 ng/well, respectively) in addition to TKβ-gal (125 ng/well) and ERET-Kluciferase (250 ng/well) into U2-OS cells grown in 24-well plates. Results are expressed relative to luciferase activity with pCDNA3 alone without 17β-estradiol. The data were reproducible and a similar pattern was obtained with transfection of HepG2 cells. c, No effect of SHP on transactivation by AR. Plasmids containing human AR with SV 40 promoter (0.05 μg/well) and SHP in CDM8 vector (0.5 μg/well) were cotransfected with TKβ-gal (0.25 μg/well) and luciferase reporter plasmid MMTV promoter (0.5 μg/well) into U2-OS cells grown in 24-well plates. Results are expressed relative to luciferase activity with empty vector alone without a ligand of AR, R1881. In all experiments, 1 μM of appropriate cognate ligands were added about 16 h after transfection, and cells were incubated for one more day before harvest for assays.

a number of nuclear receptor superfamily members (12, 16). Based on the presumption that the type I steroid receptors act as homodimers (notwithstanding the ability of ERβ to heterodimerize with ERα) the addition of ERα to the list of SHP partners is surprising.

At least superficially, the interactions of SHP with its partners appear similar to those of RXR with its partners, but with opposite consequences. In contrast to RXR, SHP generally inhibits transactivation by the superfamily members with which it interacts. Direct interaction with SHP also blocks, rather than stimulates, DNA binding by RAR/RXR heterodimers and other SHP targets (12). This latter finding led to the suggestion that the inhibitory effects of SHP are a simple consequence of the inhibition of DNA binding (12). However, further studies have demonstrated additional complexities in the interactions of SHP with its partners. Thus, the mapping results described here confirm the previous conclusion that the interaction of SHP with its partners involves sequences quite distinct from the more C-terminal subdomain that is involved in both ER homodimerization (17, 18) and RXR heterodimerization (19). When combined with the additional demonstration that SHP has the capacity to act directly as a transcriptional repressor (16), a somewhat more complex alternative mechanism for its inhibitory effects is suggested. In this model, inhibition could be a consequence of the recruitment of the SHP repressor activity to response elements via a novel SHP-receptor interaction, which would differ significantly from standard receptor homodimer or heterodimer interactions.

This latter model may account for the inhibitory effect of SHP on ERα. Steroid receptors have a potent dimerization function associated with their DNA-binding domains and are able to bind their response elements and transactivate even in the absence of their LBDs. Thus, based on the well-documented modular

nature of the ER, interaction of SHP with the ER LBD should not preclude the homodimerization of the DNA-binding domain, and therefore should not block DNA binding. Indeed, we have so far been unable to demonstrate an inhibitory effect of SHP on DNA binding by ERα *in vitro* using conditions in which binding by RAR/RXR heterodimers is strongly decreased. While this negative result may not reflect the results *in vivo*, it is most consistent with the model in which the inhibition of ERα transactivation by SHP is a consequence of the action of the SHP repressor domain, rather than an inhibition of DNA binding. This model is also supported by cotransfection results with SHPW160X, which contains the interaction domain but not the repression domain and shows little inhibitory effect on ERα transactivation (data not shown). At least one of its predictions has not been borne out, however, since we have been unable to demonstrate the existence of a multimeric DNA-ER-SHP complex using standard gel shift approaches. The similar failure to demonstrate an analogous DNA-SF1-DAX-1 complex (20) suggests either that the interactions of SHP (or the closely related DAX-1) with their receptor targets may be rather transient, or that other technical issues may preclude the identification of such complexes *in vitro*.

Regardless of the mechanism of the inhibitory effect, it could have a significant impact on ERα function. SHP is most abundantly expressed in liver and adrenal gland, with lower expression in other tissues (Refs. 12 and 21 and H-S. Choi, unpublished data). In contrast, ERα has generally been considered to function in reproductive tissues such as uterus and breast. However, ERα is also active in liver, where its expression is regulated by GH and various ligands of the nuclear hormone receptors such as $T_3$ and dexamethasone (22, 23). Recently, interest in ERα function in the liver has been significantly heightened by results suggesting that the cardioprotective effects of estrogen are based, at least in part, on its effects on liver gene expression. For example, the demonstration of ERα-mediated transactivation of low density lipoprotein (LDL) receptor promoter in transiently transfected HepG2 cells (24) suggests that direct effects of SHP on ERα transactivation could have quite significant physiological consequences. Further work will be required to identify the most important targets of ERα in the liver and the role of SHP in modulating cellular responses to estrogen.

## MATERIALS AND METHODS

### Plasmids

Construction of pT7lacHisMyc murine SHP full length and the various deletion mutants of murine SHP were reported previously (16).

The GAL4-ERα and B42-ERα were constructed by insertion of the ERα cDNA digested with *Eco*RI from pGEX-hERα LBD cDNA into pCMX-GAL4 DBD (DNA binding domain) and pJG4–5 (25) treated with *Eco*RI, respectively. ERβ was isolated from rat prostate cDNA library by PCR and inserted in pCDNA3 vector (Invitrogen, San Diego, CA). Sequencing confirmed that this isolate is identical to the ERβ clone reported previously (6).

### *In Vitro* Interaction

[$^{35}$S]methionine-labeled proteins were prepared by *in vitro* translation using pT7lacHisMyc vectors containing cDNAs coding for full-length SHP and deletion mutants (16) and the TNT-coupled transcriptional translation system with conditions as described by the manufacturer (Promega, Madison, WI). GST fusion proteins containing the LBD of human ERα or human AR, or the full-length human RXRα (GST-ER, -AR, and -RXR) were expressed in the *E. coli BL21* strain and purified using glutathione-sepharose affinity chromatography as suggested by the vendor (Pharmacia, Piscataway, NJ). *In vitro* protein-protein interaction assays were carried out as described (26).

### Yeast Two-Hybrid Assay

For the yeast two-hybrid system (25, 27), LexA-murine SHP full length and deletion (16) and B42-human ERα and -human RXRα LBD fusion plasmids were cotransformed into *Saccharomyces cerevisiae* EGY48 strain containing the β-galactosidase (β-gal) reporter plasmid, 8H18–34. Characterization of β-gal expression on plates was carried out as described (28). Similar results were obtained in more than two similar experiments with independently isolated transformants.

### Cell Culture and Transfections

HepG2 and U2-OS cells were propagated in DMEM plus 10% FBS. For transfection of HepG2 cells, cells were grown in 12-well plates with medium supplemented with 10% charcoal-stripped serum for 24 h and transfected as described (29) using the diethylaminoethyl-dextran/chloroquine method followed by dimethyl sulfoxide (DMSO) treatment. Transfections included indicated amounts of plasmids expressing proteins of interest and 0.5 μmg/well of both TKGH (30) and a reporter plasmid containing a luciferase gene and GAL4-binding elements. Approximately 16 h after DMSO treatment, 1 μM 17β-estradiol was added with fresh medium, and cells were incubated for 1 day.

For transfection of U2-OS cells, the calcium phosphate method (29) or superfect transfection reagent (Qiagen, Valencia, CA) was used with cells grown in 24-well plates. A plasmid containing a luciferase gene and vitellogenin ERE was used as a reporter and TKβ-galactosidase as the internal control plasmid. Approximately 16 h after transfection, a final concentration of 1 μM 17β-estradiol, 4-OHT, or R1881 was added with fresh medium, and cells were incubated for one more day. Luciferase was assayed as described by the manufacturer (Promega), and the results were normalized using either GH or β-gal expression from the internal control plasmids. Similar results were obtained in more than two similar experiments.

## Acknowledgments

AR mammalian expression vector and GST-AR plasmid were gifts from Dr. Z. Sun (Boston, MA).

Received February 10, 1998. Revision received June 24, 1998. Accepted October 2, 1998.

Address requests for reprints to: David D. Moore, Department of Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030. E-mail: moore@bcm.tmc.edu.

This work was supported by NIH Grants RO1 DK-46546 to D.D.M. and RO1 CA-57374 to M.B. W.S. was supported by a Helen Hay Whitney Foundation fellowship.

*Present address: Molekulargenetisches Labor der Universitäts-Frauenklinik im, BMFZ, Gebäude 23.12.04, Univeritäts skr. 1, 40225 Düsseldorf, Germany.

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